Tumor microenvironment and checkpoint molecules in primary cutaneous diffuse large B-Cell lymphoma-new therapeutic targets

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Tumor Microenvironment and Checkpoint Molecules in Primary Cutaneous Diffuse Large B-Cell Lymphoma—New Therapeutic Targets

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Abstract: Programmed death ligand 1 (PD-L1) is expressed by 20% to 57% of systemic diffuse large B cell lymphomas (DLBCLs). PD-L1 expression in primary cutaneous DLBCL (pcDLBCL) has not been studied so far. Sixteen paraffin-embedded tissue samples of pcDLBCL (13 leg type [LT], 3 others [OT]) were investigated for PD-1, PD-L1, and CD33 expression and the cellular composition of the tumor microenvironment, focusing on myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages. Membrane-bound PD-L1 expression by the tumor cells was observed in all samples, albeit to a variable extent (19.9%). As expected, most DLBCL-LT (10 cases) were classified as activated B cell like type, with a higher PD-L1 score (21.9%) compared with that of the germinal center B cell like type (7.7%). The surrounding infiltrate consisted predominately of CD163(+) M2 rather than CD68(+) macrophages (CD68:CD163 = 1:4 to 6). Moreover, a considerable proportion of CD33(+) MDSCs with PD-L1 coexpression was admixed. Tumor cells expressed CD33 to variable degrees (2% to 60%). The number of MDSCs or M2 macrophages did not correlate with pcDLBCL subtypes LT or OT. T cells were only a minor component of the tumor microenvironment. We propose that PD-L1(+) tumor cells and PD-L1(+) MDSCs shield the tumor against PD-1(+) tumor-infiltrating lymphocytes, consequently leading to inhibition and diminution of tumor-infiltrating lymphocytes. Moreover, we found a polarization to M2 macrophages, which may contribute to the poor prognosis of DLBCL patients. Thus, targeting of tumor cells and MDSCs using anti-PD-1/anti-PD-L1 or anti-CD33 antibodies might be a worthwhile new approach to treat this aggressive form of cutaneous B-cell lymphoma.

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The transmembrane receptor "programmed death-1" (PD-1) belongs to the B7/CD28 family.[1] PD-1 is expressed on the surface of activated T and B cells, follicular helper T cells, dendritic cells (DCs), and monocytes.[2,3] The interaction between PD-1 and its ligand PD-L1 reduces T-cell proliferation, cytokine release, and inhibits survival proteins (eg, bcl-xl),[4–7] which results in apoptosis.[6,7] Physiologically, PD-L1 is detected on monocytes, macrophages, DCs, and regulatory T cells (Tregs).[8] Many solid tumors and Hodgkin lymphomas express PD-L1.[9,10] In contrast, PD-L1 is only rarely expressed by non-Hodgkin lymphomas, with the notable exceptions of nodal diffuse large B-cell lymphoma (DLBCL) and virus-associated lymphoma.[11–13] Some studies demonstrated that PD-L1 expressing tumor cells can induce apoptosis in PD-1(+) tumor-infiltrating lymphocytes (TILs).[14,15] This might explain the poor prognosis of patients suffering from tumors with high PD-L1 levels compared with those whose tumors show no or low PD-L1 expression.[16–18] Moreover, inhibition of the PD-1/PD-L1 axis seems to be an effective treatment against PD-L1 expressing tumors.[10,19]

Primary cutaneous diffuse large B-cell lymphoma leg type (pcDLBCL-LT) is listed in the revised version of WHO classification (2016) as a separate entity.[20] It is characterized by a distinct histologic and immunohistochemical profile (bcl2 +, bcl-6 +/−, MUM-1 +), a typical clinical presentation with tumor nodules mostly arising on the legs of elderly women and with a 5-year survival time of $< 60\%$.[21,22] In contrast, primary cutaneous diffuse large B-cell lymphoma others (pcDLBCL-OT) refers to rare cases of large B-cell lymphomas that do not belong to the group of pcDLBCLs-LT or to the group of primary cutaneous follicle center lymphomas.[22]

The composition and function of the tumor microenvironment (TME) is one of the most important factors for both tumor survival (immune escape) and antitumoral defense. Major components of the TME comprise TILs (especially Tregs),[23,24] tumor-promoting M2 macrophages, tumor-suppressing M1 macrophages,[25] and

myeloid-derived suppressor cells (MDSCs).[26] The data on pathogenic and prognostic effects of M2 macrophages in nodal DLBCL are controversial.[27–29] Nevertheless, Shen et al[30] demonstrated that the number of M2 macrophages correlated negatively with prognosis in nodal DLBCL.

The aim of our study was to evaluate PD-1 and PD-L1 expression, and the cellular composition of the TME in pcDLBCL.

MATERIALS AND METHODS

Patients and Samples

On the basis of established diagnostic criteria,[20,21] biopsy specimens of 16 pcDLBCLs were investigated independently by 2 board-certified dermatopathologists (W.K., C.M.). Thirteen of these cases were classified as LT and 3 as OT. Fifteen samples were found to be germinal center B cell like (GCB) or activated B cell like (ABC) types according to Hans' algorithm, which is based on immunohistochemical expressions of CD10, bcl-6, and MUM-1.[31] Although it is known that these groups do not exactly correspond to molecular categories, the immunohistochemical algorithm has been recommended by the current WHO classification, due to gene expression profiling still not being a routine test.[20]

Clinical information was obtained through retrospective review of the patients' records, and communication with clinicians and patients. The patients' data were stored in a database. The Cantonal Ethics Committee of Zurich (No. 2014-0493) has approved the study.

Histology, Immunohistochemistry, and Immunofluorescence

Biopsy specimens were fixed in 10% buffered formalin, embedded in paraffin, stained with hematoxylin and eosin (H&E), and reviewed. The immunohistochemical studies were conducted using the following antibodies: PD-1 (clone MRQ-22, dilution RTU; CellMarque, Rocklin), PD-L1 (clone SP142, dilution 1:50; Roche, Mannheim, Deutschland), CD33 (clone PWS44, dilution RTU; Leica Biosystems, Muttenz, Switzerland), CD68 (clone PG-M1, dilution 1:100; Dako, Glostrup, Denmark), and CD163 (clone 10D6, dilution 1:400; Leica Biosystems). All immunohistochemical stains were performed according to the manufacturer's protocol. Double stains were performed for PD-1/PD-L1, PAX-5/PD-L1, and CD33/PD-L1. Immunofluorescence double stains for PAX-5/PD-L1 were added. Sections of human tonsils were used as positive controls.

Evaluation of the Slides

PD-L1, PD-1, and CD33 Expression by Tumor Cells

To investigate PD-L1 expression by the tumor cells, double stainings with a nuclear B-cell marker (PAX-5) were performed in all cases. Membrane-bound PD-L1 expression was examined for each sample by 1 investigator (C.M.), blinded to clinical data. As reported previously,[4] 5 high-power fields of representative tumor areas were evaluated. The expression of PD-L1 on B cells was verified by PAX-5 and PD-L1 double stains. The number of double-expressing cells was given as a rate of all B cells. The number of CD33(+) tumor cells was evaluated similarly. PD-1 expression was investigated in a single stain.

Composition and Distribution of the TME

TILs. Owing to their characteristic morphology, admixed TILs could be distinguished easily from macrophages, MDSCs, and tumor cells. The density of TILs ([0] absent, [1] focal, [2] mild, [3] moderate, and [4] severe) and their distribution pattern (peripheral vs. diffuse) were evaluated in H&E.

PD-1 and PD-L1 Expressing Cells. The density ([1] low, [2] intermediate, and [3] high) and the predominant distribution (peripheral vs. diffuse) of PD-1 and PD-L1

TABLE 1. Overview of Histologic Characteristics and the Staining Results

Case	Diagnosis: LT, OT	Subtype: ABC, GCB	Localization: 1 = Lower Limb, 2 = Upper Limb, 3 = Trunk, 4 = Head and Neck	Age at Time of Diganosis (y)	Sex: M = Male, F = Female	Expression of PD-L1 on B Cells in %	Expression of CD33 on B Cells in %	Density of TILs: 0 = Absent, 1 = Focal, 2 = Mild, 3 = Moderate, 4 = Severe
1	LT	GCB	1	77	M	7.3	66	2
2	LT	GCB	2	34	M	8	24.8	2
3	LT	ABC	1	88	M	4.7	56	2
4	LT	ABC	1	64	M	10	22	1
5	LT	ABC	1	84	M	33.82	42.7	1
6	LT	ABC	1	98	F	15.7	54	1
7	LT	ABC	1	85	M	18.5	71.6	2
8	LT	ABC	4	92	F	15.4	75.4	1
9	LT	ABC	NA	81	F	28.6	71.4	1
10	LT	ABC	1	83	F	23.8	59.7	1
11	LT	NA	1	91	F	14.9	68.1	2
12	LT	ABC	1	82	M	23.6	22.3	1
13	LT	ABC	1	90	M	44.8	78.4	2
14	OT	ABC	1	97	F	17.2	60.7	2
15	OT	GCB	3	79	M	27.5	88.6	2
16	OT	GCB	1	92	M	25.2	65.1	2

NA indicates not applicable.

expressing cells were evaluated. On the basis of PD-1/PD-L1 double stainings, the number of PD-1/PD-L1 double-expressing cells was given in percentage. The relation between PD-1 and PD-L1 expression was noted.

MDSCs and Tumor-associated Macrophages. The proportions and topographic distributions of CD33(+), CD68(+), and CD163(+) cells were recorded in 5 representative high-power fields. The number of PD-L1 expressing CD33(+) cells was evaluated in CD33/PD-L1 double stains and was depicted as a proportion of all CD33(+) cells in percentage.

Statistical Analysis

All statistical analyses were performed using SPSS 22 (IBM, Hannover, Germany). A Mann-Whitney *U* test was used to compare density and PD-1 expression of TILs, and the number of macrophages in pcDLBCL-LT versus pcDLBCL-OT. To assess inverse correlations of PD-L1(+) tumor cells and PD-L1(+)/CD33(+) cells, we calculated a 1-tailed Spearman correlation. A significance level $P < 0.05$ was applied for all tests. The small sample groups precluded statistical subgroup analyses (eg, of pcDLBCL-LT ABC vs. GCB types).

RESULTS

Patients' Characteristics and Histologic Findings

We investigated 16 biopsy specimens including 13 pcDLBCL-LT and 3 pcDLBCL-OT. Among pcDLBCL-LT tumors, 10 cases were categorized as ABC type and 2 as GCB type, and in 1 case a subtyping could not be performed, because there was not enough residual tumor material. Among the pcDLBCL-OT, 1 ABC type and 2 GCB types were found.

The mean age of the patients in pcDLBCL-LT/pcDLBCL-OT was 80.7/89 years, 9/2 were men and 4/1

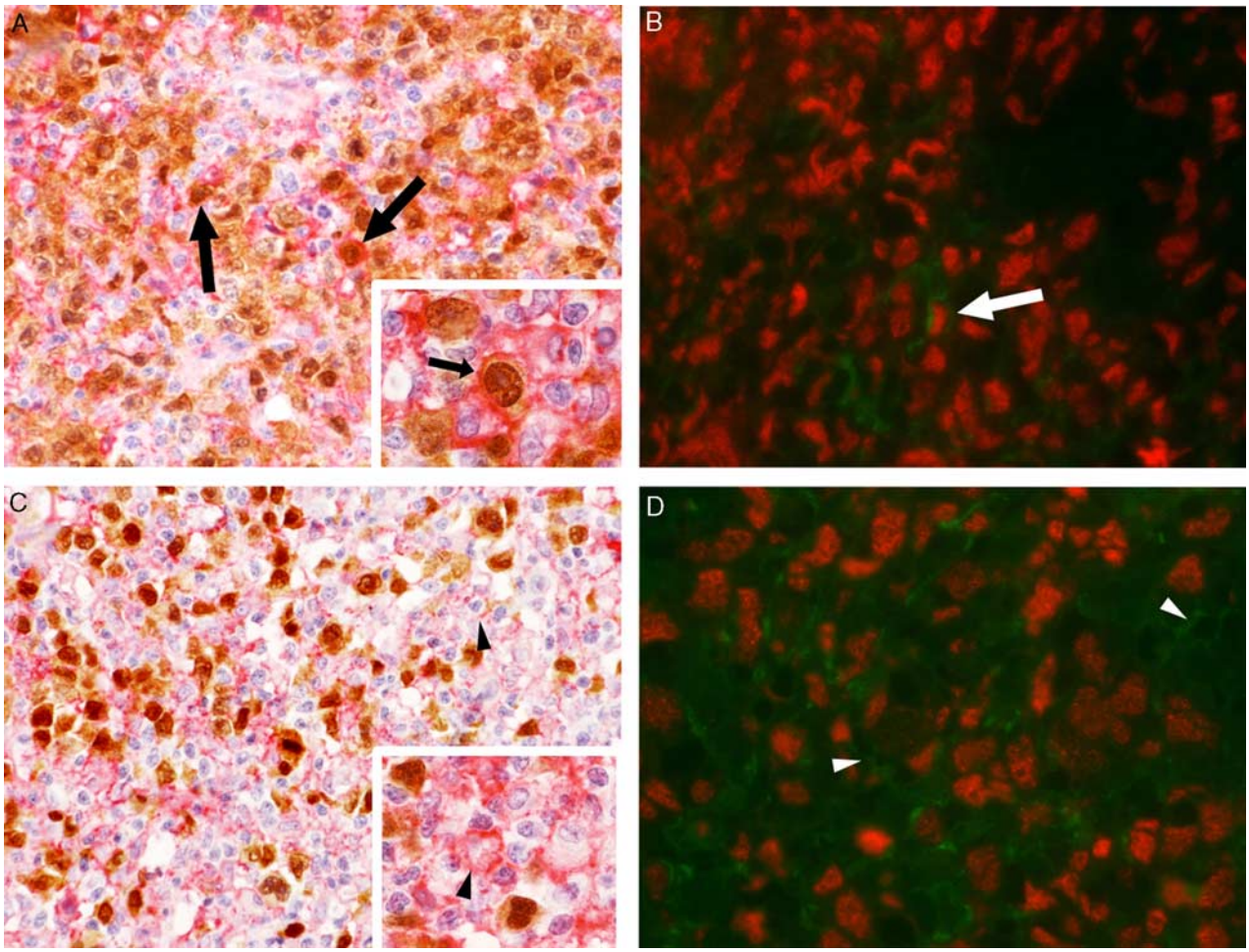

FIGURE 1. PAX-5 (brown)/PD-L1 (red) double stains. A, Central tumor area: many tumor cells presented a PAX-5/PD-L1 (arrow) double expression by immunohistochemistry. Inset: higher magnification of a PAX-5/PD-L1 double-expressing cell (arrow) (oil). B, Immunofluorescence double stains: PAX-5 (red)/PD-L1 (green) double-expressing cells (arrow). C, Peripheral tumor areas: tumor cells with PD-L1 double expression, next to a high number of PD-L1⊕ PAX-5(−) (arrow) cells by immunohistochemistry. Inset: higher magnification of a PD-L1⊕ PAX-5(−) cell (arrow) (oil). D, Immunofluorescence PAX-5 (red)/PD-L1 (green). The arrow indicates PD-L1 (green) positive cells, in which there were no B cells (Pax-5 negative).

were women. Extracutaneous disease was excluded by staging procedures according to international guidelines[32] (detailed in Table 1).

Immunohistochemical Analysis

Expression in Healthy Control

As previously reported, expression of PD-1 was found in activated T lymphocytes and follicular helper T cells.[3,33] PD-L1 was expressed by macrophages and DCs.[8] Tonsils were used as external onslide positive controls.

PD-L1, PD-1, and CD33 Expression by Tumor Cells

Distinct membrane expression of PD-L1 by the tumor cells was observed in all of our investigated samples. The mean percentage of PD-L1 expressing tumor cells (double staining with PAX-5: Figs. 1A, B) was 19.9% in pcDLBCL (range, 4.7 to 44.8, ± 10.7), 19.2% in LT (range, 4.7 to 44.8, ± 11.6), and 24.6% in OT (range, 17.2 to 27.5, ± 5.4). In PAX-5/PD-L1 double stains, a considerable number of PD-L1(+) and PAX-5(−) cells were found, which were accentuated in 3 cases at the tumor margins (Figs. 1C, D).

Among pcDLBCL-LT, the PD-L1 score was higher in ABC-type tumors (21.9) compared with that in GCB-type tumors (7.7). However, the small pcDLBCL-OT sample size precluded statistical analyses.

In both groups, PD-1 expression was lacking on most tumor cells. In pcDLBCL-LT, the tumor cells expressed CD33 in 10 of 13 samples (mean number of positive tumor cells: 15%; range, 2% to 60%) (CD33/PAX-5 double stain: Fig. 2). The mean number in tumors of the GCB type was 7.5% in contrast to 13.2% in the ABC group. About 5.7% (range, 2% to 10%) of pcDLBCL-OT tumor cells expressed CD33. There was no inverse correlation between the number of PD-L1(+) tumor cells and the number of PD-L1(+)/CD33(+) cells ($P = 0.0995$, 1-tailed Spearman). For details see Table 1.

Composition and Distribution of the TME

TILs. The distribution of TILs was diffuse in all cases, and their number was low (focal or mild) (Fig. 3A). Seven pcDLBCL-LT samples presented a focal and 6 a mild infiltrate. In pcDLBCL-OT, the infiltrate was mild in all cases. We found no significant differences between the density of TILs in pcDLBCL-LT versus pcDLBCL-OT ($P = 0.189$, Mann-Whitney U test).

PD-1 and PD-L1 Expression. The relation between PD-1 and PD-L1 was variable. In 5 cases of pcDLBCL-LT the number of PD-L1-positive cells was higher than the number of PD-1-positive cells. All of these cases were ABC types. The PD-1 expression in TILs did not differ significantly when LT and OT were compared, and it was not inversely correlated with the number of PD-L1 expressing cells. PD-1/PD-L1 double expression was found in 17% (range, 0% to 40%) of the immune cells. In 3 cases no double expression could be observed.

MDSCs and Tumor-associated Macrophages. The distribution of the immune cells (without TILs) was predominantly diffuse. In 3 cases of pcDLBCL-LT the infiltrate was accentuated in the periphery of the tumor cell conglomerates. The TME predominantly consisted of CD163(+) M2 macrophages (Fig. 3B), whereas CD68(+) macrophages were less numerous (Fig. 3D) (M1:M2 = 1:4 to 6) in all cases. Moreover, a high proportion of CD33(+) MDSCs was interspersed, which coexpressed PD-L1 in 63% of the cells (Fig. 3C).

The number of MDSCs and the number of macrophages did not correlate with the different subtypes of pcDLBCL. For details see Table 1.

The composition of the TME is illustrated in Figure 4.

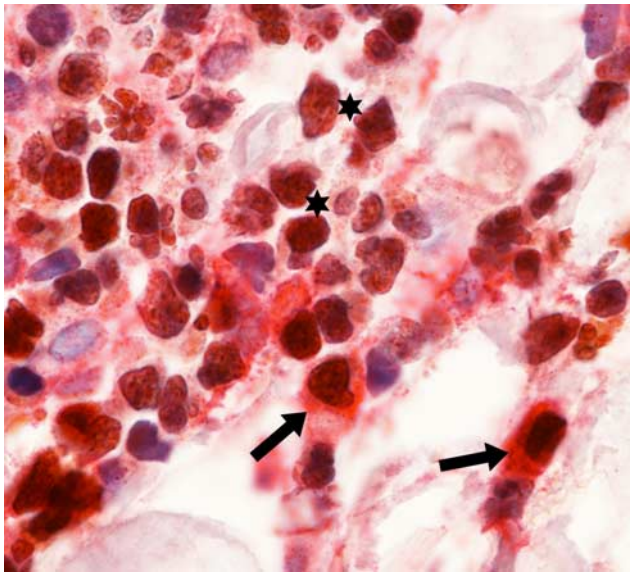

FIGURE 2. PAX-5 (brown)/CD33 (red) double stain. Brown nuclear staining of the tumor cells (PAX-5) surrounded by a cytoplasmic red signal (CD33) (arrow) (oil). For comparison, PAX-5(+) cells (brown) without cytoplasmic CD33 (red) were marked by stars.

DISCUSSION

Since the development of immune checkpoint inhibitors, such as PD-1-blocking antibodies, the expression of its ligand PD-L1 has been studied in different tumors. Hodgkin lymphoma, for example, demonstrated high levels of PD-L1 expression and an excellent response to anti-PD-1 therapy.[10] In non-Hodgkin lymphomas, PD-L1 is only rarely expressed and confined to a subset of EBV-associated B-cell lymphomas and systemic DLBCLs.[13] In the latter, PD-L1 expression was reported in 20% to 57% of cases, preferentially in the less favorable nodal DLBCL of the ABC type rather than that of the GCB type.[11–13] This discrepancy might be explained by cytogenetic alterations as well as protein expression of PD-L1/PD-L2, which is more frequent in the ABC subtype.[34] pcDLBCLs have not been investigated so far. We found PD-L1 expression in all of our samples, albeit to a variable extent. Similar to the results in nodal DLBCL,[11]

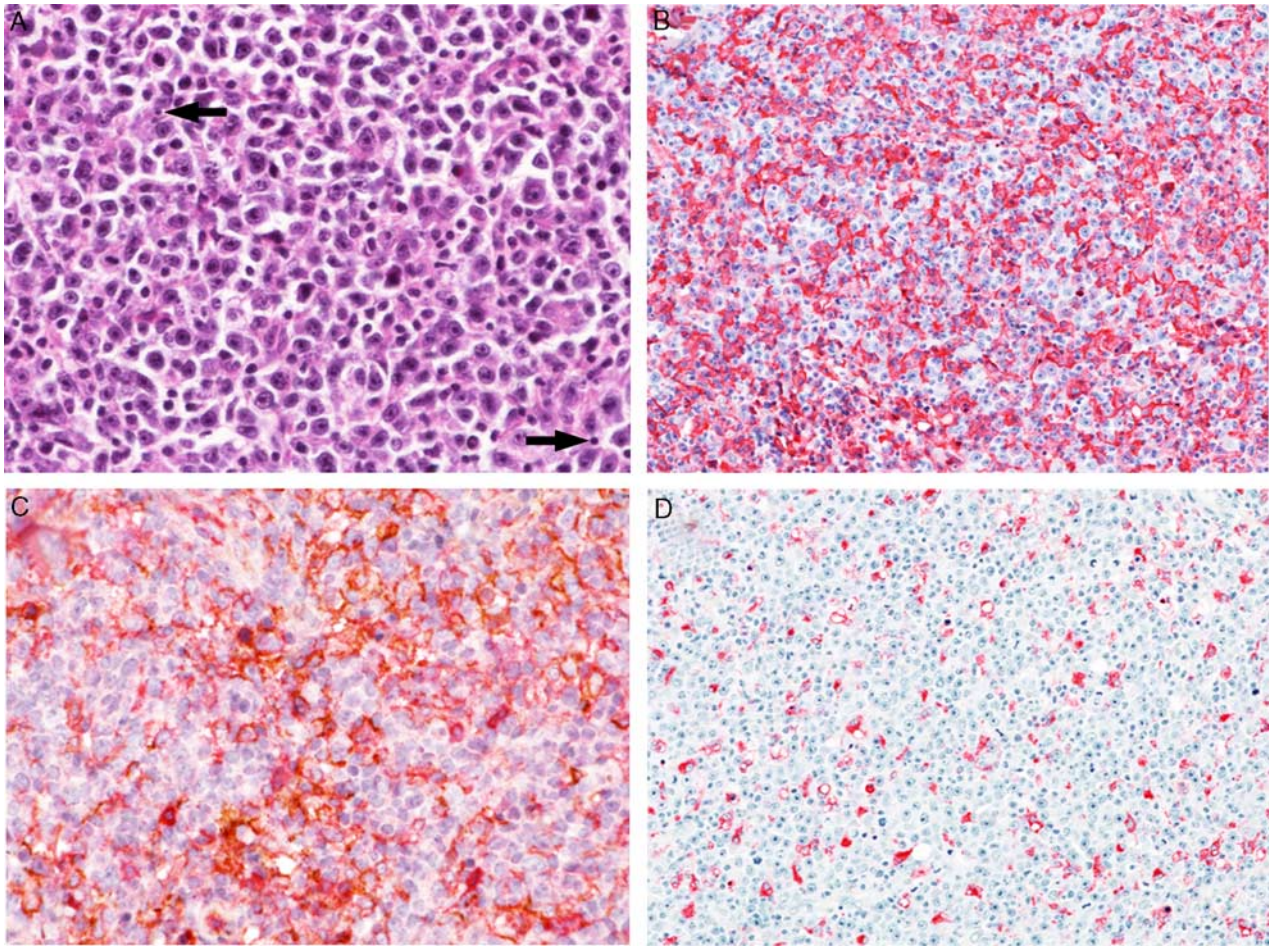

FIGURE 3. Composition of the TME. A, H&E stain demonstrated the composition of the infiltrate and stresses on the difficulty of differentiating tumor cells from macrophages and MDSCs. Only a very small number of TILs (arrow) was admixed. The microenvironment predominately consists of CD163(+) M2 macrophages (B) and CD33(+) MDSCs, which presented in 63% a PD-L1 coexpression (C). D, The number of CD68(+) M1 macrophages was low.

we could ascertain in pcDLBCL-LT a higher number of PD-L1 expressing tumor cells in the ABC-type than in the GCB-type tumors. Gene expression profile studies in pcDLBCL-LT demonstrated that most samples were similar to the ABC type of nodal DLBCL.[35] In accordance with these studies, most of our cases were classified as ABC type.

PD-1 expressing activated T lymphocytes play an important role in tumor defense,[37] which can be inhibited by PD-L1 expressing B-cell lymphomas.[11] We recently demonstrated a significantly lower level of PD-1 expressing TILs in pcDLBCL-LT as compared with both indolent primary cutaneous marginal zone lymphoma and primary cutaneous follicle center lymphoma.[33] Consistent with our current data, it is reasonable to assume that PD-L1 expressing tumor cells induce apoptosis in PD-1(+) TILs and stop the attraction of additional immune cells. This might, at least in part, explain the low level of PD-1(+) TILs in pcDLBCL-LT. In contrast, PD-L1 was not expressed in systemic marginal zone lymphoma and follicle center lymphoma.[12] In light of the known functions of PD-1/PD-L1[36] together with our expression profile, it is conceivable that the expression of PD-1/PD-L1 will be a promising target in the therapy of aggressive pcDLBCL.

Interestingly, even tumors with no or low PD-L1 expression by the tumor cells themselves can respond to PD-1 inhibition.[4,5,37] This is arguably due to a high number of PD-L1(+) cells within the TME, which shields the tumor against attacking TILs. Tregs, for example, can inhibit PD-1-positive TILs[38,39] through PD-L1 expression. In pcDLBCL, we have confirmed and extended previous results[40] of low numbers of T cells in the TME. This observation is also in line with a previous report[41] suggesting that Tregs do not play a primary role in the microenvironment of pcDLBCL. They found low numbers of TILs in PD-L1-positive tumors and discussed that PD-1(+) TILs are expected to be low in the ABC type and more abundant in the GCB type, because in normal tissue PD-1 is highly expressed in germinal center follicular helper T cells.

MDSCs are a heterogenous population of immature myeloid cells, representing precursors of granulocytes,

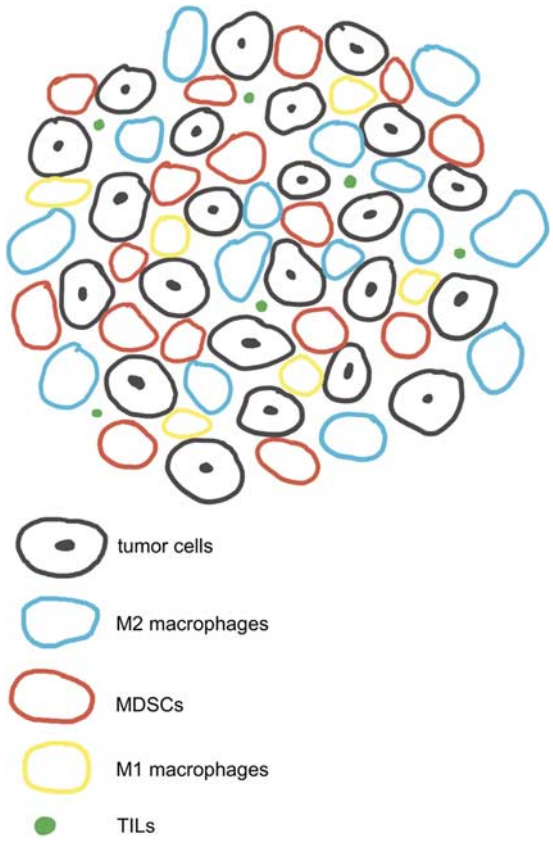

FIGURE 4. Illustration of the composition of the TME in pcDLBCL. The tumor cells (black) are interlaced with a high number of M2 macrophages (blue) and MDSCs (red). M1 macrophages were less numerous (yellow). Only a few TILs were found (green).

macrophages, and DCs. Human MDSCs are characterized as $HLADR^{-/low}CD11b^{+}CD14^{-}CD15^{+}CD33^{+}$ granulocytic origin (G-MDSC) and $HLADR^{-/low}CD11b^{+}CD14^{+}CD15^{-}CD33^{+}$ monocytic origin (M-MDSC).[42–44] In systemic DLBCL an enrichment of MDSC has been described.[45] In contrast to a predominance of G-MDSC in solid tumors,[46–48] in lymphomas both populations (G-MDSC and M-MDSC) are increased and suppress T-cell responses.[48–51] Our data are in line with these observations. We postulate that next to the PD-L1(+) tumor cells themselves, PD-L1(+) MDSCs suppress T-cell functions. Our study also opens up the possibility to target MDSCs therapeutically. Humanized antibodies directed against CD33, for example, can block MDSCs and were already tested for the treatment of acute myeloid leukemia.[52] The expression of CD33 has never been reported before in mature B-cell lymphomas and is an interesting new finding. Therefore, an inhibition of CD33 can simultaneously also attack the tumor cells directly.

Studies of gene-expressing profiles in nodal DLBCL have revealed a high number of M2 macrophages in the TME.[53,54] Macrophages can shift their functional phenotype depending on signals generated from tumor and stromal cells. On the basis of their function, they can be divided into M1 and M2 macrophages. M1 macrophages are involved in inflammatory responses and antitumoral defense.[55] In contrast, M2 macrophages reduce inflammation and have tumor-promoting effects.[55] High numbers of M2 macrophages in nodal DLBCL were associated with a poor clinical outcome.[30] We found a polarization to M2 macrophages in all of our pcDLBCL cases. As previously reported,[57] we hypothesized that the shift toward M2 macrophages was induced by the tumor cells or their microenvironment. Moreover, M2 macrophages and tumor cells can release cytokines (eg, IL-10 and TGF-β) that suppress T-cell functions directly or indirectly through PD-L1 expression[56] and can promote the M2 polarization.[57]

In summary, tumor cells of pcDLBCL consistently express PD-L1 and CD33. The TME consists predominately of M2 macrophages. T cells and CD68(+) macrophages are only a minor component. Admixed MDSCs expressed PD-L1 in 63% of the cells. We postulate that PD-L1(+) tumor cells/cells of the TME induce apoptosis in PD-1(+) TILs, which might explain the lower level of PD-1 + TILs in pcDLBCL. We postulate that PD-L1(+) cells of the microenvironment shield the tumor against attacking TILs. Therefore, direct inhibition of tumor cells through PD-L1 and CD33 as well as inhibition of microenvironmental cells, especially MDSCs, are a new and promising therapeutic option.

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